Composition of *Dithyrea wislizenii* fruit extract and free-radical scavenging activity of its constituents

Sabine Montaut, Julie Grandbois, Laura S. Rossi, Sonia Kamal, James Khouri, Melissa G. Ménard, and Hélène A. Joly

Abstract: Glucolesquerellin (**2**), glucohesperin (**3**), quercetin 3-*O*-sophoroside (**4**), and quercetin 3-*O*-sophoroside-7-*O*-glucoside (**5**), isolated from the fruit of *Dithyrea wislizenii*, were quantified by HPLC. The fruit extract and flavonoids were not found to be toxic by using a brine shrimp lethality assay. The fruit extract and the flavonoids and glucosinolates were submitted to a free-radical scavenging activity assay with the diphenylpicrylhydrazyl radical (DPPH$^{\bullet}$). The concentration of quercetin (**6**) (a positive control for the flavonoids) able to scavenge 50% of DPPH$^{\bullet}$ (SC_{50}) was 32 ± 2 μmol/L (or 4 ± 1 μg/mL), which was about 27 times more potent than the crude extract. Compounds **4** and **5** had a SC_{50}, the concentration of the compound required to scavenge 50% of the DPPH$^{\bullet}$, of 78 ± 1 μmol/L and 113 ± 10 μmol/L, respectively. The positive control for the glucosinolates, glucoraphasatin, (**1**) had a SC_{50} of 1768 ± 60 μmol/L. The glucosinolates **2** and **3** had a SC_{50} of 7819 ± 1968 and 970 ± 63 μmol/L, respectively.

Key words: Dithyrea wislizenii, Brassicaceae, flavonoids, glucosinolates, free-radical scavengers.

Résumé : La glucolesquerelline (**2**), la glucohespérine (**3**), la quercétine 3-*O*-sophoroside (**4**), et la quercétine 3-*O*-sophoroside-7-*O*-glucoside (**5**), isolées à partir des fruits de *Dithyrea wislizenii*, ont été quantifiés par HPLC. L'extrait de fruit et les flavonoïdes n'ont pas montré de toxicité en utilisant le test de mortalité de la crevette de saumure. L'extrait de fruit, les flavonoïdes et les glucosinolates ont été soumis à un test d'activité anti-radicalaire à l'aide du radical diphénylpicrylhydrazyle (DPPH$^{\bullet}$). La concentration de quercétine (**6**) (un contrôle positif pour les flavonoïdes) capable de piéger 50 % de DPPH$^{\bullet}$ (SC_{50}) était 32 ± 2 μmol/L (ou 4 ± 1 μg/mL), ce qui était environ 27 fois plus puissant que l'extrait brut. Les composés **4** et **5** avaient des SC_{50} de 78 ± 1 μmol/L et 113 ± 10 μmol/L, respectivement. Le contrôle positif pour les glucosinolates, la glucoraphasatine, (**1**) avait une SC_{50} de 1768 ± 60 μmol/L. Les glucosinolates **2** et **3** avaient des SC_{50} de 7819 ± 1968 et 970 ± 63 μmol/L, respectivement.

Mots-clés : Dithyrea wislizenii, Brassicaceae, flavonoïdes, glucosinolates, piégeurs de radicaux libres.

Introduction

The antioxidant properties of plant extracts originating from members of the Brassicaceae family were first attributed to polyphenolic compounds.[1] However, there have been reports that nonphenolic compounds can also contribute to the antioxidant activity of some *Brassica* sp.[2] In fact, the glucosinolate (GL), 4-methylsulfanyl-3-butenylGL (**1**), was shown to have free-radical scavenging activity towards the radical cation 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS$^{\bullet+}$) and the diphenylpicrylhydrazyl (DPPH$^{\bullet}$) free radical.[3,4]

Dithyrea wislizenii Engelm. (Brassicaceae) (syn. *Dimorphocarpa wislizenii* (Engelm.) Rollins), an endemic annual plant found in Nevada, Colorado, Utah, Arizona, New Mexico, and Texas, grows in open sandy soil of dry grasslands and deserts. This branched or unbranched, greyish, hairy plant has pinnately lobed leaves, white flowers in dense, thick racemes, and is commonly referred to as "spectacle pod".[5] Many Native American tribes used this plant as food for animals,[6] as a drug to reduce swelling, to treat delirium, on wounds, and in miscellaneous ceremonies.[7]

In previous phytochemical studies, the fatty acid content of the seed oil of *D. wislizenii* was investigated.[8,9] In addition, a GC analysis of the hydrolysis products of the compounds extracted from the seeds showed the presence of 5-methylsulfanylpentyl-, 6-methylsulfanylhexyl-, 7-methylsulfanylheptyl-, 5-methylsulfinylpentyl-, and 6-methylsulfinylhexyl isothiocyanates (ITCs), which suggests that the seeds contain the parent GLs.[10] Recently, our group analyzed the fruit extract of *D. wislizenii* for desulfoGLs and intact GLs using HPLC–APCI-MS and HPLC–ESI-MS, respectively. 2-PropenylGL (sinigrin) was shown to be present in the extract. 6-MethylsulfanylhexylGL (glucolesquerellin, **2**), 6-methylsulfinylhexylGL (glucohesperin, **3**), 7-methylsulfanylheptylGL, and 5-methylsulfanylpentylGL (glucoberteroin) were isolated from the extract and characterized by UV, IR, and NMR spectroscopies, and mass spectrometry.[11] Finally, the indole alkaloid, dithyreanitrile (7-methoxy-α,α-bis(methylsulfanyl)-1*H*-indole-3-acetonitrile), was isolated from the seeds of

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D. wizlizenii. This compound was shown to inhibit the feeding of the fall armyworm (*Spodoptera frugiperda* Smith) and the European corn borer (*Ostrinia nubilalis* Hbn.) larvae.[12]

Here we report the isolation, structure elucidation, and quantification of two flavonoids present in the fruit extract of *D. wislizenii*. In addition, we have quantified the two major GLs (**2** and **3**) in the extract. The toxicity on brine shrimp larvae of the fruit extract and of the flavonoids was probed. The free-radical scavenging activity of the fruit extract and of the isolated flavonoids was evaluated by electron spin resonance (ESR) spectroscopy. Seeing that some GLs have been implicated in the free-radical scavenging activity of plant extracts, but not many have been studied, we determined the free-radical scavenging activity of the GLs found in the fruit extract of *D. wislizenii*.

Results and discussion

To use the plant extract in health-benefit applications or in animal and human nutrition, its lack of lethality was probed against *Artemia salina* L. (brine shrimp) larvae. The brine shrimp lethality test showed that the fruit extract was not lethal against *A. salina* larvae at the tested concentrations.[13] This was expected, since this plant was given to animals as food.[6]

To test free-radical scavenging activity, the *D. wislizenii* fruit extract was added to solutions containing a known concentration of the stable free radical, DPPH•. The average amount of *D. wislizenii* fruit extract able to scavenge 50% of the DPPH• (SC_{50}) was 110 ± 11 μg/mL (Table 1). We decided to determine which natural products contributed to this activity; hence, we have isolated the major secondary metabolites in the extract and probed their free-radical scavenging activity.

Several peaks were detected in the HPLC chromatogram of the extract of *D. wislizenii* fruit (Fig. S1, Supplementary data), using the experimental conditions described in the literature.[14] In addition to the previously reported occurrence of sinigrin, glucohesperin, glucoberteroin, glucolesquerellin, and 7-methylsulfanylheptylGL,[11] the plant extract contained two compounds eluting at retention times (t_R) of 36.8 min (compound **4**) and 29.8 min (compound **5**). These two compounds were isolated from the extract using the chromatographic separations outlined in the Experimental section. The spectroscopic data of compound **4** were in agreement with those published in the literature for the 3-*O*-β-D-(2-*O*-β-D-glucopyranosyl)glucopyranoside of quercetin, also known as quercetin 3-*O*-sophoroside (**4**)[15–19] (Fig. 1; Table S1, Supplementary data) and the data of compound **5** were in agreement with those published in the literature for quercetin 3-*O*-sophoroside-7-*O*-glucoside (**5**) (Fig. 1; Table S1, Supplementary data).[15–17,20,21]

Although **4** and **5** have been found in several members of the Brassicaceae family, it is the first time that they have been observed in the *Dithyrea* sp. For example, quercetin 3-*O*-sophoroside has been previously identified in the genus *Brassica*[22] and in watercress,[23] and quercetin 3-*O*-sophoroside-7-*O*-glucoside has been found in canola and tronchuda cabbage.[19–21,24–28]

The major compounds found in the *D. wislizenii* extract, namely **2**–**5** were quantified by HPLC. The quantities of **2**, **3**, **4**, and **5** in the plant extract were estimated to be 12.4, 6.0, 2.1, and 18.0 μmol/g of dried fruit, respectively.

The free-radical scavenging activities of the natural products isolated from *D. wislizenii* were tested (Table 1). Quercetin (**6**) and glucoraphasatin (**1**), compounds known to have free-radical scavenging activity against DPPH•, were used as the positive control for the flavonoids and GLs isolated from *D. wislizenii*, respectively. The SC_{50} of **6** has been expressed in both μg/mL and μmol/L to allow for comparison with the activity of the crude extract and the isolated flavonoids and GLs. The SC_{50} of compound **6** is 27 times smaller than that of the crude extract of *D. wislizenii*. This means that compound **6** scavenges DPPH• better than the crude extract. This is not surprising as the extract is a complex mixture of compounds, many of which do not possess free-radical scavenging activity. Flavonoids **4** and **5** have weaker free-radical scavenging activities than **6** (Table 1). Compound **6** is 2.5 times better at scavenging free-radicals than **4**, and 3.6 times better than **5**. This finding is consistent with the trolox equivalent antioxidant capacity (TEAC) values obtained for **6** and **4**. The TEAC value of **6** was reported to be 3 times higher than that of **4**.[1] Furthermore, in our investigation, **4** was found to be 1.5 times better at scavenging free-radicals than **5**, which means that the latter had the weakest free-radical scavenging activity among the flavonoids tested.

The three main structural features in flavonoids that determine their overall free-radical scavenging activity are (*i*) the *o*-dihydroxyl groups in the B ring, (*ii*) the double bond between C(2) and C(3) in the C ring, which is conjugated to the 4-oxo group (C=O) in the C ring, and (*iii*) the presence of additional OH groups at positions 3 and 5.[29] The presence of these three main structural features in **6** explains why it possesses a high free-radical scavenging activity. The substituents at positions 3 and 7 are glucose residues that affect the ability of the B-ring hydroxyl groups to donate one hydrogen in **4** and **5**.

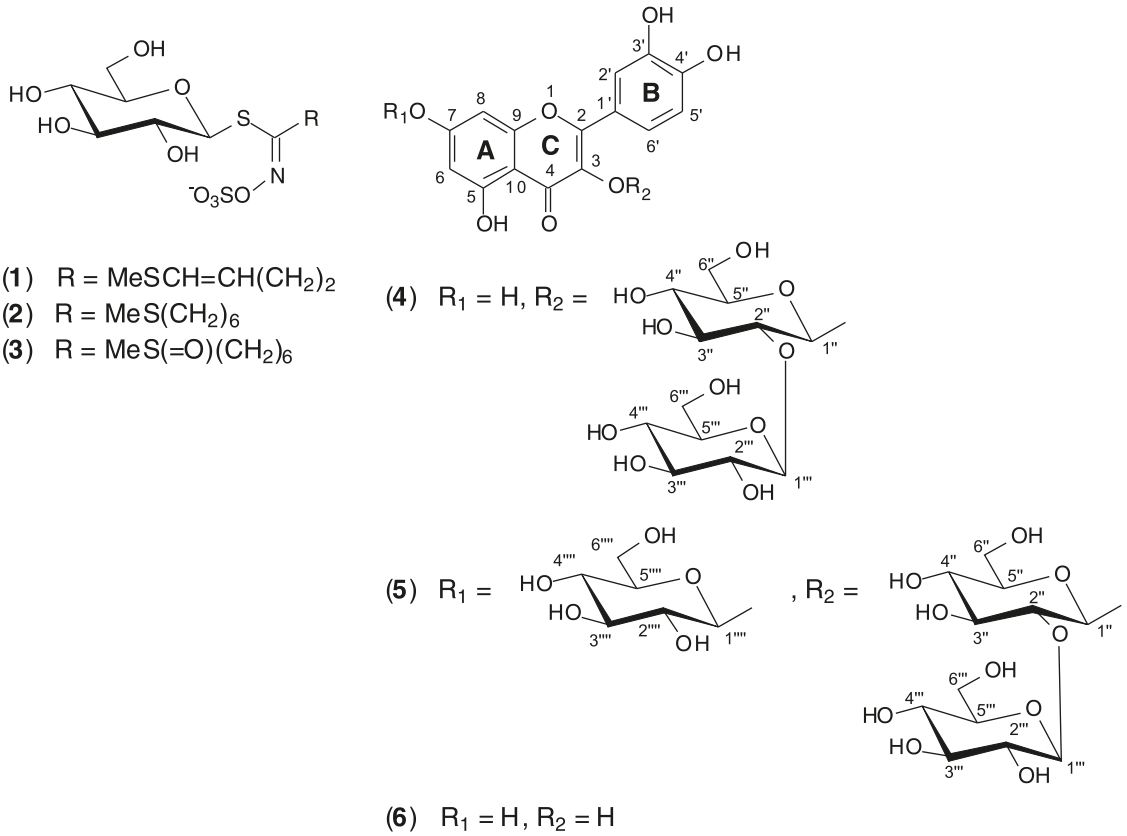
The SC_{50} of **1** was found to be 4 times smaller than that of **2**, whereas the SC_{50} of **3** was 2 times lower than the SC_{50} of **1** and 8 times smaller than that of **2**. This means that **3** is a better free-radical scavenger than **1** and that **1** is a better scavenger than **2** (Table 1). The SC_{50} of **2** has a relatively large standard deviation because it demonstrated relatively weak free-radical scavenging activity. Although the mechanism for the reaction of DPPH• with GLs is not known, Barillari and co-workers[4] proposed that the reaction of DPPH• with 4-methylsulfanyl-3-butenyl ITC, also called raphasatin, the degradation product of **1**, proceeds via two pathways (Fig. 2). These involve abstraction of the hydrogen atom α to the allylic functional group, –CH=CHSCH$_3$, (path 1) and abstraction of the hydrogen atom that is α to the –N=C=S (path 2). Presumably, –CH=CHSCH$_3$ and –N=C=S are responsible for the weakening of the respective C–H bonds. Abstraction of a second hydrogen in both of the proposed pathways leads to a conjugated ITC. In the case of the intact GLs used in the present study, one could propose a similar mechanism (Fig. 3). The hydrogen atoms most likely to react with the DPPH• are those α to the *O*-sulfated *Z*-thiohydroximate functional group as well as those α to –CH=CHSCH$_3$ (for **1**), SCH$_3$ (for **2**), and SOCH$_3$ (for **3**). For **2** and **3**, the sulfanyl and sulfinyl groups are separated from the *O*-sulfated

Table 1. Free-radical scavenging activities of the fruit extract of *Dithyrea wislizenii*, glucolesquerellin (**2**), glucohesperin (**3**), quercetin 3-*O*-sophoroside (**4**), and quercetin 3-*O*-sophoroside-7-*O*-glucoside (**5**), against diphenylpicrylhydrazyl (DPPH•).

Extract/product	DPPH[a]	Average SC_{50} ± SD (% error)[b]
Quercetin (**6**)	160	4±1 μg/mL (21.8)
Dithyrea wislizenii fruit extract	160	110±11 μg/mL (10.2)
Quercetin (**6**)	350	32±2 μmol/L (7.6)
Quercetin 3-*O*-sophoroside (**4**)	350	78±1 μmol/L (1.3)
Quercetin 3-*O*-sophoroside-7-*O*-glucoside (**5**)	350	113±10 μmol/L (9.1)
Glucoraphasatin (**1**)	350	1768±60 μmol/L (3.4)
Glucolesquerellin (**2**)	350	7819±1968 μmol/L (25.2)
Glucohesperin (**3**)	350	970±63 μmol/L (6.5)

Note: SC_{50}, the concentration of the compound required to scavenge 50% of the DPPH•.
[a]Concentration of DPPH• in the capillary in μmol/L.
[b]Tested in triplicate from three independent experiments.

Fig. 1. Structure of glucolesquerellin (**2**), glucohesperin (**3**), quercetin 3-*O*-sophoroside (**4**), and quercetin 3-*O*-sophoroside-7-*O*-glucoside (**5**) isolated from *Dithyrea wislizenii*.



Z-thiohydroximate functional group by six methylene groups. The effect of the *O*-sulfated *Z*-thiohydroximate functional group, if any, on the α-C–H bond strength would be comparable. Therefore, the difference in the SC_{50} must be primarily dependent on the SCH_3 and $SOCH_3$ groups. The sulfinyl group is more electron-withdrawing than the sulfanyl group, which makes the adjacent hydrogen atoms in **3** more acidic than in **2**.[30] Furthermore, the S=O bond will delocalize the unpaired electron over the π bond. This allows **3** to donate the hydrogen atoms easily, resulting in a higher free-radical scavenging activity than those of **2** and **1**. For **1**, the C=C bond is electron-withdrawing and will make the adjacent hydrogen atom more acidic and easier for the free radical to abstract. Moreover, the C=C bond allows the free electron to be delocalized over the π bond. This results in a higher free-radical scavenging activity than that of **2**. Our observations suggest that the presence of a sulfinyl group and C=C bond contributes to the overall scavenging activity of certain GLs. Papi et al.[4] studied the free-radical scavenging activity of raphasatin and its intact GL (**1**) towards DPPH• and found the ITC to be 3 times more active than the intact GL. This suggests that the ITC group is the major contributor to this

Fig. 2. Proposed mechanisms of the reaction of 4-methylsulfanyl-3-butenyl isothiocyanate with diphenylpicrylhydrazyl radical (DPPH•).[4]

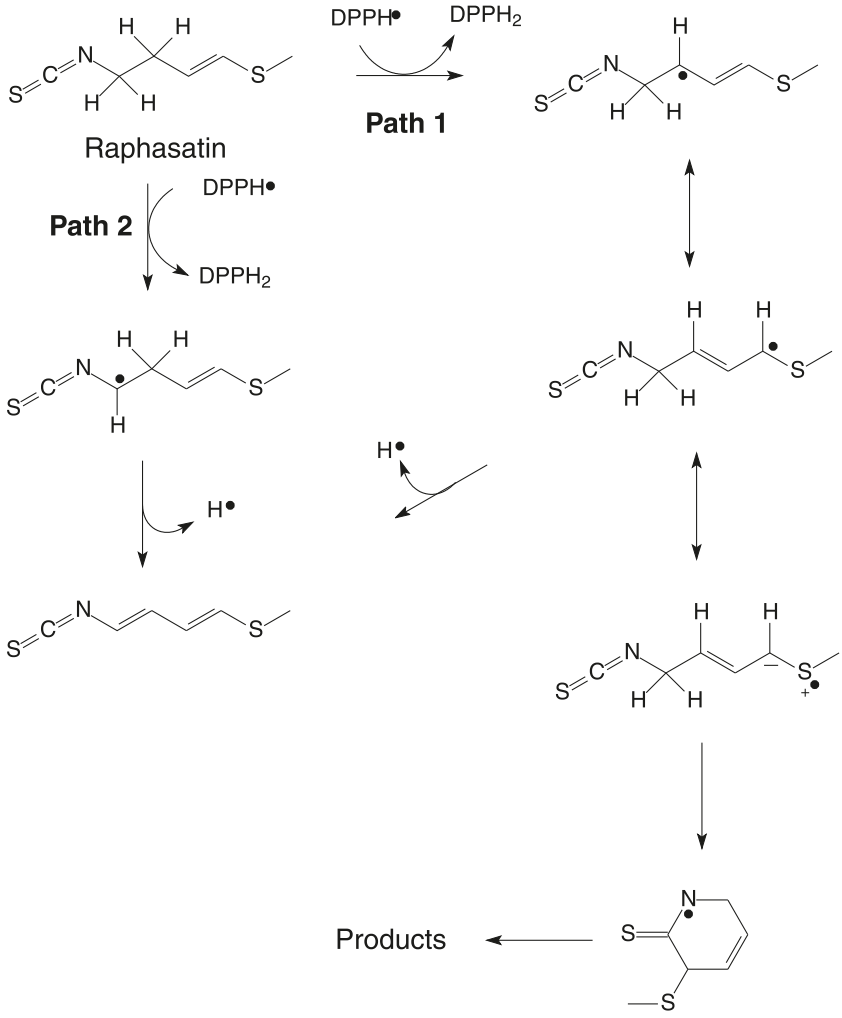
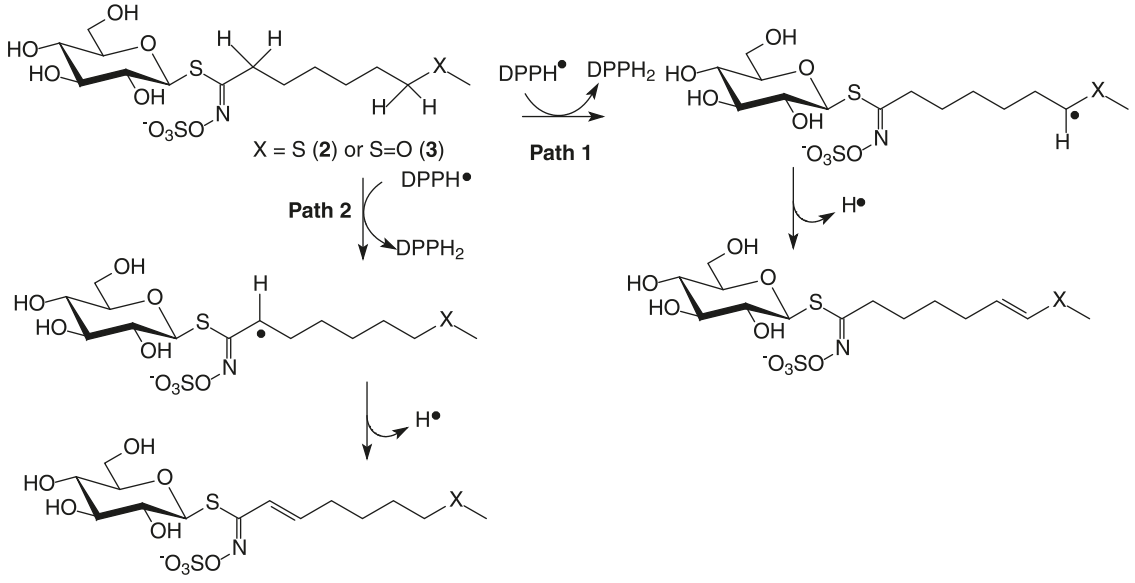


Fig. 3. Proposed mechanisms of the reactions of glucolesquerellin (**2**) and glucohesperin (**3**) with diphenylpicrylhydrazyl radical (DPPH•).



increased activity. Because **3** was found to be twice as active as **1**, we expect that its corresponding ITC hesperin would be twice as active as raphasatin.

It is worth noting that other natural products, such as the ones in garlic containing a sulfoxide functional group such as alliin and thiosulfinates such as allicin, have also been shown to possess some free-radical scavenging properties.[31] Finally, all the GLs we tested were weaker free-radical scavengers than the flavonoids we analyzed. Glucohesperin (**3**), the best GL among the ones we tested, was 8.6 times less active than **5**, the poorest free-radical scavenger among the flavonoids we tested. Overall, our results indicated that the flavonoids strongly contributed to the free-radical scavenging activity of *D. wislizenii* fruit extract, whereas the GLs contributed to the activity to a lesser extent.

Conclusions

In this study, we have isolated and identified quercetin 3-*O*-sophoroside (**4**) and quercetin 3-*O*-sophoroside-7-*O*-glucoside (**5**) representing the first report of the presence of flavonoids in a *Dithyrea* species. The crude extract, **4**, and **5** were not lethal against *A. salina* at the concentrations tested. Both isolated flavonoids and GLs contributed to the free-radical scavenging activity of *D. wislizenii*. It was revealed that the flavonoids were stronger free-radical scavengers than the GLs and, hence, the major contributors to the free-radical scavenging activity of *D. wislizenii*. From the structures of the flavonoids, we confirmed that the more glycosylated the flavonoid, the weaker its free-radical scavenging activity. From the structures of the GLs, we proposed that the presence of the sulfanyl and sulfinyl groups would contribute to the free-radical scavenging activity of the tested GLs. Further investigations of the free-radical scavenging activity of the plant extract and of the major natural products isolated should be carried out against the superoxide anion ($O_2^{\bullet -}$) and hydroxyl radical ($HO^{\bullet}$). Such a study could contribute to understanding the anti-inflammatory properties of the plant metabolites (most probably the flavonoids) initially observed by many Native North American tribes who used the plant to reduce swelling. Because the plant extract and its components display free-radical scavenging ability, other studies could be directed towards probing the potential cancer-preventive effects of *D. wislizenii*. Furthermore, the free-radical scavenging activity of lesquerellin and hesperin, the ITCs of **2** and **3**, could be investigated as they are expected to be better scavengers than their parent GLs. In fact, raphasatin, the glucoraphasatin ITC, has been shown to be 3 times more potent as a scavenger of $DPPH^{\bullet}$ than glucoraphasatin.[4] In conclusion, *D. wislizenii* is a good source of free-radical scavengers.

Experimental

Materials

All solvents were ACS grade and used as such except for $CHCl_3$, which was redistilled. All deuterated solvents were purchased from Cambridge Isotopes Laboratories, Inc. (Andover, Massachusetts). Formic acid, L-(+)-arabinose, D-(+)-galactose, D-glucose, D-(+)-xylose, NaOH, and EtOAc were purchased from BDH (Toronto, Ontario). HPLC-grade MeOH, absolute EtOH, and triethylamine (reagent grade) were purchased from Fisher Scientific (Whitby, Ontario). Sinigrin, D-(+)-mannose, DMSO, NaOAc, and quercetin dihydrate were purchased from Sigma-Aldrich (Oakville, Ontario). $DPPH^{\bullet}$ was purchased from Sigma-Aldrich and stored at 5 °C. Glacial AcOH was purchased from Anachemia Canada Inc. (Montreal, Quebec). HCl was purchased from Stanchem (Etobicoke, Ontario). H_3BO_3 was bought from Matheson, Coleman, and Bell Manufacturing Chemists (Norwood, Ohio). $AlCl_3$ was from the McArthur Chemical Co., Ltd. (Montreal, Quebec). Isopropanol and thymol were purchased from EM Science (Gibbstown, New Jersey). HPLC-grade H_2O was generated in the laboratory through a Nanopure Diamond Ultrapure water system by Barnstead (Dubuque, Iowa). Kieselgel 60 F_{254} analytical TLC aluminum sheets were purchased from EM Science; compounds were visualized under UV light and by dipping the plates in sulfuric thymol solution containing 1% (*w*/*v*) thymol, 10% (*v*/*v*) H_2SO_4, and 95% EtOH, followed by heating. Flash column chromatography (FCC) was carried out using 70–230 mesh 60 Å normal-phase silica gel (Sigma-Aldrich). Sephadex LH-20 was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). C-18 silica gel was purchased from Alltech (Guelph, Ontario). C-18 silica gel cartridges (Mega Bond Elut Flash, 10 g sorbent mass, 60 mL volume) were obtained from Varian, Inc (Mississauga, Ontario).

Instrumentation

UV spectra were recorded on a Biochrom Ultrospec 2100 pro UV–vis spectrophotometer (Cambridge, UK). Specific rotations ($[\alpha]_D$) were measured with a Rudolph Research Corporation Autopol II automatic polarimeter (Flanders, New Jersey) at ambient temperature using a 20 cm path length cell; the units are 10^{-1}deg cm^2 g^{-1} and the concentrations (*c*) are reported in grams per 100 millilitres. IR spectra were recorded with a PerkinElmer Paragon 1000PC IR spectrometer (Waltham, Massachusetts) or a Bruker Optics Tensor 27 FTIR instrument (Boston, Massachusetts) using KBr disks. NMR spectra were recorded on a Bruker Avance spectrometer at 600 MHz (^{1}H) and 150 MHz (^{13}C) at the Saskatchewan Structural Science Centre (Saskatoon, Saskatchewan), or on a Bruker DRX 500 spectrometer at 500 MHz (^{1}H) and 125 MHz (^{13}C) at Laurentian University (Sudbury, Ontario); δ values were referenced to D_2O at 4.80 ppm and to DMSO-d_6 at 2.50 ppm for ^{1}H NMR spectra and at 39.5 ppm for ^{13}C NMR spectra. High-resolution electrospray mass spectrometric (HR-ESI-MS) measurements were recorded using an API Qstar XL mass spectrometer at the Saskatchewan Structural Science Centre. A Varian E109 EPR spectrometer operating at X-band was used to measure the free-radical scavenging activity of the antioxidants. ESR spectra were recorded at room temperature (21–23 °C) at centre field, 3257; field sweep, 100 G; and microwave power, 2 mW. For a given experiment, the receiver gain was kept constant.

Plant material

Dry *Dithyrea wislizenii* fruit was obtained from the Plants of the South West company (Santa Fe, New Mexico). A voucher (No. 21023) was deposited at the Department of Biology Herbarium, Laurentian University. Cherry Belle radish

seeds (Gardener's choice) were purchased from a local store in Sudbury, Ontario.

HPLC-ESI-MS analysis

Dithyrea wislizenii extract (50 mg) was dissolved in 2 mL of MeOH and filtered through a plug of cotton prior to analysis by HPLC. Analysis was performed by injecting a 20 μL aliquot of the solution of crude extract into an Agilent Technologies HP 1100 (New Castle, Delaware) HPLC equipped with a quaternary pump, automatic injector, diode-array detector (wavelength range 190–600 nm), degasser, and a Hypersil ODS column (5 μm, 4.6 mm × 200 mm). The two mobile-phase solvents, MeOH and H_2O, were prepared with 0.15% triethylamine and 0.18% formic acid added as ion-pairing reagents. Both solutions were filtered using 0.45 μm nylon membranes. The initial mobile phase was 100% HPLC-grade H_2O. At 10 min, the mobile phase was switched to a linear gradient of 100% H_2O to 100% MeOH over 60 min.[14] After each run, the initial mobile-phase conditions were set and the system was allowed to equilibrate. The flow rate was kept constant at 1 mL/min. The column temperature was held at room temperature. The HPLC was interfaced to an Agilent model 6120 MS with a Chemstation data system (LC–MSD B.03.01). The ES interface was a standard ES source operating with a capillary voltage of 4 kV and temperature of 350 °C. The system was operated in the negative and positive ion ES modes. Nitrogen was used as nebulizing and drying gas at a flow of 10 L/min (35 psig, 1 psi = 6.894 kPa). The MS was programmed to perform full scans between *m/z* 100 and 1000 amu.

Extraction and isolation

Dithyrea wislizenii fruits (347 g) were frozen in liquid N_2, ground in a mortar, and immediately extracted 3 times with boiling MeOH (1.4 L for the first extraction and 1.05 L for the other extractions) for 30 min. The methanolic solution was filtered and concentrated to dryness (33 g). This extract was dissolved in H_2O (200 mL) and submitted to liquid–liquid extractions with solvents of increasing polarity, $CHCl_3$, EtOAc, and BuOH. Each organic layer was concentrated ($CHCl_3$ fraction, 15.2 g; EtOAc fraction, 1.2 g; and BuOH fraction, 6.1 g) and the aqueous layer was lyophilized (11.7 g). The BuOH fraction (3.6 g) was fractionated by FCC (normal phase; gradient $CHCl_3$/MeOH 8:2, 7:3, and 0:10 *v/v*; 25 mL fractions). Fractions 22–35, obtained from the FCC column, eluted with $CHCl_3$/MeOH 7:3 (*v/v*), were combined (210 mg) and fractionated on a Sephadex LH-20 column (H_2O/MeOH 10:0 and 0:10 *v/v*, 5 mL fractions). Fractions eluted with MeOH were combined and yielded quercetin 3-*O*-sophoroside (**4**, 64.6 mg). Fractions 126–177, obtained from the FCC (normal phase) mentioned previously, eluted with MeOH, were combined (611 mg) and subjected to Sephadex LH-20 column (H_2O/MeOH 10:0 and 0:10 *v/v*, 5 mL fractions) fractionations. Fractions 4–8, eluted with H_2O from the Sephadex LH-20 column, were combined (175 mg) and submitted to a solid-phase separation (C18 cartridge; H_2O/MeOH 100:0, 95:5, 90:10, 80:20, and 0:100 *v/v*; 5 mL fractions). Fractions 69–82, eluted with H_2O/MeOH 80:20 (*v/v*), yielded quercetin 3-*O*-sophoroside-7-*O*-glucoside (**5**, 56.6 mg).

Acid hydrolysis of flavonoids and identification of aglycones and carbohydrates

The flavonoid was mixed with 6% aq HCl (5 mL). The solution was heated for 45 min. After cooling, the solution was extracted 3 times with EtOAc (3 × 5 mL). Evaporation of the aqueous layer yielded the sugars that were analysed by TLC (isopropanol/AcOH/H_2O, 3:1:0.5 *v/v/v*). The carbohydrate composition was determined according to the TAPPI test method T249-cm-85 by FPInnovations-Paprican division (Pointe-Claire, Quebec).[17] Arabinose, xylose, mannose, galactose, and glucose were used as reference compounds. The organic layer, after drying over sodium sulfate, was evaporated. Quercetin was identified in this layer by LC–MS analysis by comparison of the t_R, UV, and mass spectra with those of a commercial standard.

Quercetin 3-O-sophoroside (4H-1-benzopyran-4-one, 2-(3,4-dihydroxyphenyl)-3-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]-5,7-dihydroxyflavone; 4)

Yellow amorphous powder. HPLC, t_R = 36.8 min. $[\alpha]^{26}_D$ – 147 (*c* 0.08, MeOH). UV (MeOH) λ_{max} (nm, log ε): 206 (4.5), 258 (4.2), 356 (4.1); + NaOH: 273, 408; + $AlCl_3$: 275, 429; + $AlCl_3$ + HCl: 270, 355, 390; + NaOAc: 270, 350; + NaOAc + H_3BO_3: 259, 358. FTIR (KBr, cm^{-1}): 3408 (OH), 2927 (CH), 2341, 1656 (α,β-unsaturated C=O), 1607, 1507, 1455, 1361, 1305, 1198, 1168, 1075 (C-O). For ^1H and ^{13}C NMR data see Table S1. HR-ESI$^-$-MS *m/z* calcd for $C_{27}H_{29}O_{17}^-$: 625.1410; found: 625.1406 [M – H]$^-$. HR-ESI$^+$-MS *m/z* calcd for $C_{27}H_{31}O_{17}^+$: 627.1556; found: 627.1573 [M + H]$^+$.

Quercetin 3-O-sophoroside-7-O-glucoside (4H-1-benzopyran-4-one, 2-(3,4-dihydroxyphenyl)-3-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]-7-(β-D-glucopyranosyloxy)-5-hydroxyflavone; 5)

Yellow amorphous powder. HPLC, t_R = 29.8 min. $[\alpha]^{22}_D$ – 98 (*c* 0.02, MeOH). UV (MeOH) λ_{max} (nm, log ε): 206 (4.5), 256 (4.2), 356 (4.0); + NaOH: 275, 418; + $AlCl_3$: 274, 295, 334, 436; + $AlCl_3$ + HCl: 268, 351, 400; + NaOAc: 263, 352; + NaOAc + H_3BO_3: 261, 354. FTIR (KBr, cm^{-1}): 3416 (OH), 2923 (CH), 2360, 2341, 1656 (α,β-unsaturated C=O), 1600, 1492, 1271, 1203, 1174, 1074 (C-O). For ^1H and ^{13}C NMR data see Table S1. HR-ESI$^-$-MS *m/z* calcd for $C_{33}H_{39}O_{22}^-$: 787.1938; found: 787.1909 [M – H]$^-$.

Purification of glucoraphasatin (1)

Cherry Belle radish seeds were soaked for 1 min in 1% sodium hypochlorite and rinsed with distilled H_2O. Seeds were laid on two sheets of filter paper in Petri dishes and were incubated at room temperature (23 ± 2 °C) under an 8 h day / 16 h night photoperiod. The sprouts were watered twice a day with H_2O. Fresh nine-day-old Cherry Belle radish seedlings (899 g) were frozen in liquid N_2, ground in a mortar, and immediately extracted 3 times with boiling MeOH (3 × 3 L) for 30 min. The methanolic solution was filtered and concentrated to dryness (35 g). This extract was dissolved in H_2O (100 mL) and subjected to liquid–liquid extractions with solvents of increasing polarity, EtOAc and BuOH. Each organic layer was concentrated (EtOAc fraction, 1.1 g; and BuOH fraction, 3.4 g) and the aqueous layer was dried (29.4 g). The aqueous fraction (3 g) was purified using column

chromatography (C18 reverse phase, gradient H_2O/MeOH 10:0 and 0:10 *v*/*v*, 23 mL fractions). Fractions 18–84, eluted with H_2O from the C18 reverse-phase column, were combined (232 mg) and subjected to solid-phase separation (C18 cartridge, H_2O/MeOH 10:0 and 0:10 *v*/*v*, 3 mL fractions). Fractions 7–34, eluted with H_2O yielded glucoraphasatin (**1**, 101 mg). The purity of the amorphous solid **1** was assessed by HPLC, and the compound was characterized by ^1H and ^{13}C NMR spectroscopies. The spectroscopic data were consistent with the ones previously published.[3]

Purification of glucolesquerellin (2) and glucohesperin (3)

These GLs were isolated and characterized as previously reported.[11]

Flavonoid and glucosinolate quantification

Several solutions of **2** and **3** (5×10^{-3}, 10^{-3}, 2×10^{-4}, and 4×10^{-5} mol/L), **4** (9.8×10^{-4}, 4.9×10^{-4}, and 2.45×10^{-4} mol/L), and **5** (10^{-2}, 5×10^{-3}, 10^{-3} mol/L) were prepared via serial dilution in MeOH. Each solution was injected (20 µL) into the HPLC. Calibration curves were then constructed using Microsoft Excel from data obtained from three independent experiments run in triplicate.

Brine shrimp lethality assay

Brine shrimp eggs were obtained from a local store and were hatched in artificial sea water. The hatching, harvesting, and dispensing of nauplii were performed in a manner similar to that of Solis et al.[13] *Artemia salina* eggs were hydrated in a 200 mL beaker containing artificial sea water (table salt, 38 g/L). Eggs were sprinkled into the darkened half of the beaker and separated from the aerated, illuminated other half by a plastic divider. The hatching time was ~48 h. *Artemia salina* nauplii that accumulated in the illuminated part of the beaker were transferred into a 96 well microplate (VWR International Inc., Mississauga, Ontario) (10 nauplii per 100 microlitres of artificial sea water per well). *Dithyrea wislizenii* fruit extract, **4**, and **5** were added to live nauplii and, after a 24 h exposure time at 22 °C, the brine shrimp lethality was measured by counting the number of dead (nonmotile) nauplii per well, using a binocular microscope. One hundred microlitres of MeOH were then added to each well and, after 15 min, the total number of shrimp in each well was counted. The crude extract was tested at 125, 250, and 500 ppm. Compounds **4** and **5** were tested at 125, 250, 500, and 1000 µmol/L. Sea water was used as the control. A positive assay was the death of all brine shrimp with 100 µmol/L of thymol. The results were obtained from three independent experiments in triplicate.

DPPH• assay

Solution preparation

The SC_{50} for **6** (positive control for flavonoids), **1** (positive control for GLs), and compounds **2**–**5** were extracted from the linear regression line of plots of % scavenging activity (SA) vs compound concentration (µmol/L). The SC_{50} was determined from the linear regression line. SC_{50} values represent the mean of three independent experiments, run in triplicate, and are expressed as the mean ± SD, calculated using Microsoft Excel. The solutions tested for SA using the ESR method outlined as follows were prepared by the serial dilution of stock solutions of **1**–**6**. EtOH was used as the solvent in the dilutions. A 981.5 µmol/L quercetin (**6**) stock solution was used to prepare five solutions with concentrations between 12.6 and 196.3 µmol/L. A 6900 µmol/L stock solution of **4** in DMSO was used to prepare four solutions with concentrations between 22.8 and 284.3 µmol/L. A 172 µmol/L stock solution of **5** in DMSO was used to prepare four solutions with concentrations between 22 and 275 µmol/L. A 7129 µmol/L stock solution of **1** in DMSO was used to prepare four solutions with concentrations between 1643 and 4277 µmol/L. A stock solution (about 26 227 µmol/L) of **2** was prepared in DMSO. From the stock solution, three serial dilutions in EtOH resulted in solution concentrations between 7 787 and 22 958 µmol/L. The stock solution and the three solutions were tested for free-radical scavenging activity. A 3147 µmol/L stock solution of **3** in DMSO was used to prepare three solutions with concentrations ranging from 201 to 1259 µmol/L. These as well as the stock solution were tested for free-radical scavenging activity.

General procedure

In general, 100 µL of a 700 µmol/L DPPH• solution was transferred to a 4 mL vial (Supelco 2-7138) containing a stirring bar. The antioxidant solution (100 µL) being tested was added to the vial. The timer was started and the vial was capped and placed on the stir plate. The reaction was carried out at room temperature (21–23 °C). At the 30 min mark, a portion of the contents of the vial were transferred with a glass syringe (Hamilton 80801) to a 3 cm long capillary tube (fabricated by cutting a Kimble No. 345059 (VWR) capillary tube to the desired length). The capillary tube was sealed with paraffin. The capillary was placed in an ESR tube (Wilmad), which was subsequently inserted in the cavity of the Varian X-band ESR spectrometer. The ESR spectrum was recorded at a frequency of ~9120 MHz and a microwave power of 2 mW. The center field and field sweep were set at 3257 and 100 G, respectively. The area under the ESR spectrum of DPPH• was determined by using the double integration (DI) feature in the ESR software known as EPRware (Scientific Software Services, Northville, Michigan). The DI is related to the concentration of DPPH• in the tube. The %SA is defined as shown in eq. [1].

$$[1] \qquad \%SA = 100(1 - DI_S/DI_{NC})$$

DI_S and DI_{NC} are the double integration of the DPPH• spectrum in the presence and absence, respectively, of the compound being tested for the free-radical scavenging activity.

Supplementary data

Supplementary data (Fig. S1 and Table S1) are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/v2012-042.

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